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ORIGINAL ARTICLE

# Characterization of migratory primordial germ cells in the aorta-gonad-mesonephros of a 4.5-week-old human embryo: a toolbox to evaluate *in vitro* early gametogenesis

**Maria Gomes Fernandes[1,†], Monika Bialecka[1,†], Daniela C. F. Salvatori[2,*], and Susana M. Chuva de Sousa Lopes [1,3,*]**

[1]Department of Anatomy and Embryology, Leiden University Medical Center, Leiden 2333-ZC, The Netherlands [2]Central Laboratory Animal Facility, Leiden University Medical Center, Leiden 2333-ZC, The Netherlands [3]Department for Reproductive Medicine, Ghent University Hospital, Ghent 9000, Belgium

*Correspondence address. Department of Anatomy and Embryology, Leiden University Medical Center, Leiden 2333-ZC, The Netherlands. Tel: +31-71-526-9350; Fax: +31-71-526-8289; E-mail: lopes@lumc.nl orcid.org/0000-0003-3866-2803

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**STUDY QUESTION:** Which set of antibodies can be used to identify migratory and early post-migratory human primordial germ cells (hPGCs)?

**STUDY FINDING:** We validated the specificity of 33 antibodies for 31 markers, including POU5F1, NANOG, PRDM1 and TFAP2C as specific markers of hPGCs at 4.5 weeks of development of Carnegie stage (CS12–13), whereas KIT and SOX17 also marked the intra-aortic hematopoietic stem cell cluster in the aorta-gonad-mesonephros (AGM).

**WHAT IS KNOWN ALREADY:** The dynamics of gene expression during germ cell development in mice is well characterized and this knowledge has proved crucial to allow the development of protocols for the *in vitro* derivation of functional gametes. Although there is a great interest in generating human gametes *in vitro*, it is still unclear which markers are expressed during the early stages of hPGC development and many studies use markers described in mouse to benchmark differentiation of human PGC-like cells (hPGCLCs). Early post-implantation development differs significantly between mice and humans, and so some germ cells markers, including SOX2, SOX17, IFITM3 and ITGA6 may not identify mPGCs and hPGCs equally well.

**STUDY DESIGN, SIZE, DURATION:** This immunofluorescence study investigated the expression of putative hPGC markers in the caudal part of a single human embryo at 4.5 weeks of development.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** We have investigated by immunofluorescence the expression of a set of 33 antibodies for 31 markers, including pluripotency, germ cell, adhesion, migration, surface, mesenchymal and epigenetic markers on paraffin sections of the caudal part, including the AGM region, of a single human embryo (CS12–13). The human material used was anonymously donated with informed consent from elective abortions without medical indication.

**MAIN RESULTS AND THE ROLE OF CHANCE:** We observed germ cell specific expression of NANOG, TFAP2C and PRDM1 in POU5F1+ hPGCs in the AGM. The epigenetic markers H3K27me3 and 5mC were sufficient to distinguish hPGCs from the surrounding somatic cells. Some mPGC-markers were not detected in hPGCs, but marked other tissues; whereas other markers, such as ALPL, SOX17, KIT, TUBB3, ITGA6 marked both POU5F1+ hPGCs and other cells in the AGM. We used a combination of multiple markers, immunostaining different cellular compartments when feasible, to decrease the chance of misidentifying hPGCs.

**LARGE SCALE DATA:** Non-applicable.

[†]These authors contributed equally.

**LIMITATIONS REASONS FOR CAUTION:** Material to study early human development is unique and very rare thus restricting the sample size. We have used a combination of antibodies limited by the number of paraffin sections available.

**WIDER IMPLICATIONS OF THE FINDINGS:** Most of our knowledge on early gametogenesis has been obtained from model organisms such as mice and is extrapolated to humans. However, since there is a dedicated effort to produce human artificial gametes *in vitro*, it is of great importance to determine the expression and specificity of human-specific germ cell markers. We provide a systematic analysis of the expression of 31 different markers in paraffin sections of a CS12–13 embryo. Our results will help to set up a toolbox of markers to evaluate protocols to induce hPGCLCs *in vitro*.

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**Key words:** human / primordial germ cells / migration / antibodies / expression / pluripotency / epigenetics / surface markers / aorta-gonad-mesonephros

# Introduction

The dynamics of gene expression during specification and further development of primordial germ cells (PGCs) in mouse is well characterized (Saitou and Yamaji, 2012; Bertocchini and Chuva de Sousa Lopes, 2016; Saitou and Miyauchi, 2016; Tang *et al.*, 2016). Consequently, markers (including antibodies) to identify and facilitate FACS-sorting of differentiated PGC-like cells (PGCLCs) from mouse pluripotent stem cells (PSCs) *in vitro*, as well as to evaluate the efficiency of *in vitro* differentiation protocols, are well known and reliably used. This useful toolbox of antibodies has been crucial for the success of recent protocols using mouse PSCs to recapitulate gametogenesis *in vitro* (Hayashi *et al.*, 2011, 2012; Hikabe *et al.*, 2016). In humans, the dynamics of gene expression is less well studied and in fact it is still unclear when PGCs are specified (Bertocchini and Chuva de Sousa Lopes, 2016; Tang *et al.*, 2016). This lack of knowledge is hampering the efficient benchmark of differentiation protocols recapitulating gametogenesis *in vitro* using human PSCs (Clark *et al.*, 2004; Bucay *et al.*, 2009; Kee *et al.*, 2009; Gkountela *et al.*, 2013; Irie *et al.*, 2015; Tilgner *et al.*, 2008; Sasaki *et al.*, 2015; Sugawa *et al.*, 2015).

In mouse, PGC precursors (pPGCs) express PRDM1 (or BLIMP1), TFAP2C (or AP2gamma) and PRDM14 (Bertocchini and Chuva de Sousa Lopes, 2016; Tang *et al.*, 2016) to suppress the somatic program and become lineage restricted as PGCs around embryonic day (E)7.2 (Tam and Zhou, 1996; de Sousa Lopes *et al.*, 2007). From this stage on and until they undergo meiosis, mPGCs also express key genes associated with pluripotency such as POU5F1 (or OCT4) (Kehler *et al.*, 2004), NANOG (Chambers *et al.*, 2007), SOX2 (Campolo *et al.*, 2013), DPPA3 (or STELLA) (Payer *et al.*, 2003), SALL4 (Yamaguchi *et al.*, 2015) and ALPL (or TNAP) (MacGregor *et al.*, 1995).

Mouse transgenic PSCs, such as *Blimp1::mvenus* and *Stella::ecfp* (Hikabe *et al.*, 2016; Zhou *et al.*, 2016) have proved useful to optimize protocols for the differentiation of mPSCs to mPGCLCs. Hence, mPGCLCs FACS-sorted for SSEA1+ and ITGB3+ and subsequently co-cultured with either E12.5 female gonads or with newborn testis (a necessary step to induce meiosis), were able to undergo respectively oogenesis or spermatogenesis *ex vivo*, generating functional gametes (Hikabe *et al.*, 2016; Zhou *et al.*, 2016). Human transgenic PSCs for *Blimp1::tdtomato*, *Tfap2c::egfp* (Sasaki *et al.*, 2015) and *Nanos3::mcherry* (Irie *et al.*, 2015) have facilitated differentiation to human PGCLCs (hPGCLCs), but these do not upregulate late PGC markers or undergo meiosis.

Much of what is known regarding human early gametogenesis, in particular PGC specification, relies heavily on extrapolation from studies in mouse (Bertocchini and Chuva de Sousa Lopes, 2016; Tang *et al.*, 2016), monkey (Sasaki *et al.*, 2016) and pig (du Puy *et al.*, 2011; Kobayashi *et al.*, 2017). Although several studies have tested antibody-markers of pluripotency and germ cells in histological sections of human foetal gonads (Gaskell *et al.*, 2004; Pauls *et al.*, 2006; Anderson *et al.*, 2007; Gkountela *et al.*, 2013; Heeren *et al.*, 2015, 2016; Kerr *et al.*, 2008a, b; Rajpert-De Meyts *et al.*, 2004), only few report the analysis of migratory hPGCs (Mollgard *et al.*, 2010; Mamsen *et al.*, 2012). Moreover, studies on hPGCs have highlighted differences in marker expression and hence gametogenesis between mice and humans.

Despite recent advances in hPGC single-cell transcriptomics (Guo *et al.*, 2015; Li *et al.*, 2017) and differentiation protocols from hPSCs to hPGCLCs, many of the markers that are currently used to access differentiation and to sort pure populations of hPGCs/hPGCLCs are not uniquely expressed in hPGCs and their expression has not been validated in earlier stages of human development. In this study, we evaluated and validated for the expression of several pluripotency- and PGC-associated markers/antibodies in migratory and early colonizing hPGCs in one single human embryo of Carnegie stage 12–13 (CS12–13). Our results showed the specificity of a panel of 31 markers to distinguish hPGCs, crucial to evaluate hPGCLC differentiation *in vitro*.

# Materials and Methods

## Ethical approval for use of human foetal tissue

All procedures conformed to the Declaration of Helsinki for Medical Research involving Human Subjects and were approved by the Medical Ethical Committee of the Leiden University Medical Center (P08.087). The embryo was donated for research with informed consent from elective abortions without medical indication.

## Collection and sex genotyping of human foetal material

The developmental age of the embryo was determined by ultrasonography. The embryo was isolated in cold 0.9% NaCl (Fresenius Kabi, Zeist,

the Netherlands) and fixed in 4% paraformaldehyde (PFA) (Merck, Darmstadt, Germany) overnight (o/n) at 4°C, washed 3× in phosphate-buffered saline without Ca++ and Mg++ (PBS0) and stored in 70% ethanol at 4°C.

The sex was determined by genomic PCR for Amelogenin (AMELX/AMELY), that distinguishes the X and Y chromosomes by amplicon size (977 bp and 790 bp, respectively) as described (Heeren *et al.*, 2015). The primers used were: FW 5′-CTG ATG GTT GGC CTC AAG CCT GTG-3′ and RV 5′-TAA AGA GAT TCA TTA ACT TGA CTG-3′; the PCR programme used was 5 min 95°C, 34× (1 min 95°C, 30 s 60°C, 2 min 72°C), 10 min 72°C, and the PCR products were run on a 1.5% agarose gel.

## Immunofluorescence in paraffin sections

The W4.5 embryo was embedded in paraffin using a Shandon Excelsior tissue processor (Thermo Scientific, Altrincham, UK) and sectioned (5 µm) using a RM2065 microtome (Leica Instruments GmbH, Wetzlar, Germany) onto StarFrost slides (Waldemar Knittel, Braunschweig, Germany). Human foetal material (W8–9 gonad, W9 mesonephros, W16–18 kidney, W19 adrenal, W19 placenta and W15 colon) were isolated, embedded in paraffin and processed for immunofluorescence. Immunofluorescence was performed as described (Heeren *et al.*, 2015). Briefly, paraffin sections were deparaffinised in xylene, rehydrated through an ethanol series and finally water, followed by antigen retrieval in 0.01 M citric buffer (pH 6.0) for 12 min at 98°C on a microwave (TissueWave 2, Thermo Scientific) and allowed to cool down. After being rinsed in PBS0, sections were treated for 1 h at room temperature (RT) with blocking solution (1% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, USA), 0.05% Tween-20 (Merck-Schuchardt, Hohenbrunn, Germany) in PBS0). Thereafter, sections were incubated with primary antibodies diluted in blocking solution overnight at 4°C, washed three times for 20 min at RT with PBS0 and incubated with the respective secondary antibodies for 2 h at RT. Primary antibodies and dilutions used, as well as used matching isotypes used as negative controls, are listed in Supplementary Table S1. Secondary antibodies and dilutions used are listed in Supplementary Table S2. Results from the isotype controls (negative controls) are presented in Supplementary Fig. S1.

## Immunofluorescence of whole mounts

Human W8–9 gonads were cut transversally in several smaller pieces (12–15 pieces) using a scalpel (Swann Morton, Sheffield, England). These small pieces were permeabilized in 0.2% Triton-X100 (Merck, Darmstadt, Germany) in PBS0 for 20 min at RT and blocked in a solution of 1% BSA (Life Technologies, Carlsbad, USA) and 10% foetal calf serum (Life Technologies, Carlsbad, USA) in PBS0 for 1 h at RT. The gonadal pieces were then incubated with primary antibodies (Supplementary Table S1) diluted in 1% BSA in PBS-T (0.1% Tween-20 (Merck, Darmstadt, Germany) in PBS0) overnight at 4°C, washed twice with PBS-T, incubated with secondary antibodies (Supplementary Table S2) diluted in 1% BSA/PBS-T o/n at 4°C, washed twice with PBS-T and once with MilliQ water, and counterstained with DAPI (Life Technologies, Carlsbad, USA). Samples were mounted on StarFrost slides using ProlonGold.

## Teratoma assay

Paraffin sections of teratomas were a gift from D. Salvatori. The formation of teratoma was ethically approved by the Animal Ethical Committee of the Leiden University Medical Center (DEC 13165) and previously described (Bouma *et al.*, 2017). Briefly, adult male mice (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ, Charles River) were subcutaneously injected with 2102Ep cells ($1 \times 10^6$ cells per injection) in the flank region. The tumour growth was monitored periodically and, when reaching a volume of 2 $cm^3$, was isolated, embedded in paraffin blocks and used for immunofluorescence and as described above. Antibodies (and dilutions) used are listed in Supplementary Tables S1 and S2.

## Imaging

Bright field images of the embryo were made using a Tablet-PC PET W1010 I0NL (Peaq, Oberursel, Germany). Fluorescence images were made on an inverted Leica TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) using the Leica Application Suite Advanced Fluorescence software (LAS AF, Leica). Different channels were acquired sequentially and the merged imaged was generated afterwards. Colour settings were performed in Fiji (Schindelin *et al.*, 2012) and figures were assembled in Adobe Photoshop CC (Adobe Systems, San Jose, CA, USA) and Adobe Illustrator CC (Adobe Systems, San Jose, CA, USA).

# Results

## Morphological characteristic of the human embryo analysed

In 1948, Emil Witschi performed a detailed histological analysis of 23 serially sectioned embryos (ranging from 3.5 to 8 mm) from the Carnegie collection, and generated a graphical reconstruction of the migratory trajectory of the hPGCs based on morphology (Witschi, 1948). The quantification of the hPGCs in a 4.2 mm embryo revealed that, at that stage, most hPGCs had left the gut endoderm and 71% were migrating thought the mesentery and rounding the coelomic angle heading for either the left or right gonadal primordium (Witschi, 1948).

We have analysed a rare (and almost intact) embryo of 4 weeks and 5 days gestation (Fig. 1A), corresponding to CS12–13 (Hill, 2017). We counted 30 somites, but the most posterior part of the embryo was missing and therefore, the somite number was likely higher. Sections through the caudal part of the embryo, containing the aorta-gonad-mesonephros (AGM) region, revealed the gut, dorsal mesenterium, mesonephros, dorsal aorta, somites, notochord, neural tube and surface ectoderm (Fig. 1B). These are important landmarks to evaluate the specificity of the antibodies tested. The thickening of the gonadal primordia had not formed yet (Fig. 1B), suggesting that most hPGCs were still actively migrating.

## Early hPGCs showed a distinct epigenetic state from the somatic compartment

We used POU5F1 (or OCT4) to mark hPGCs unambiguously and observed hPGCs migrating through the dorsal mesentery and rounding the coelomic angle to reach the gonadal primordia (Fig. 2A). POU5F1 showed strong nuclear localization but was also visible in the cytoplasm.

The sex of the embryo (XX) was confirmed by immunostaining for histone 3 lysine 27 trimethylation (H3K27me3), as the characteristic perinuclear accumulation of H3K27me3, corresponding to the silent chromosome X in somatic cells, was visible (Geens and Chuva De Sousa Lopes, 2017). By contrast, in hPGCs H3K27me3 coated the entire nuclear envelope and this was in fact sufficient to distinguish hPGCs from the surrounding somatic cells (Fig. 2A, Supplementary Fig. S2A). As described in late hPGCs (Gkountela *et al.*, 2013), early

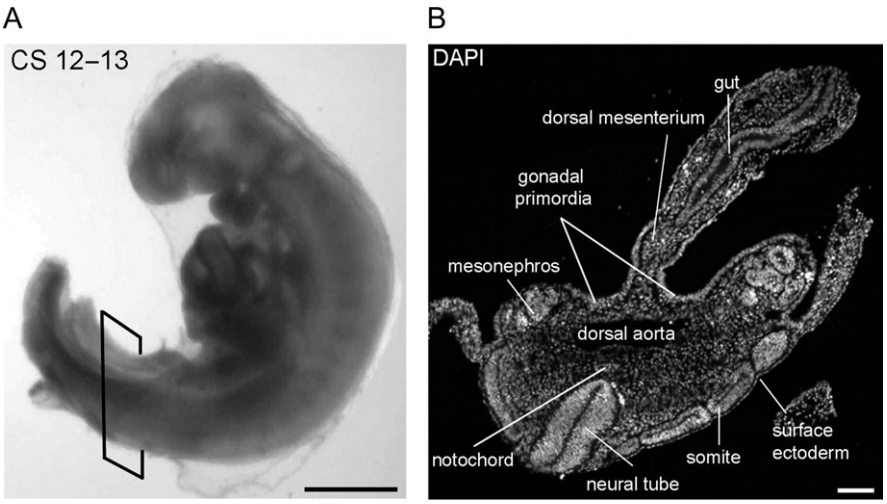


**Figure 1** Morphological characteristics of a CS12–13 human embryo. (**A**) Bright field image of a human embryo with 4 weeks and 5 days of development, corresponding to Carnegie stage (CS)12–13. The caudal part of the embryo, containing the aorta-gonad-mesonephros (AGM) region was sectioned (black square shows the orientation). (**B**) Histological section of the embryo with several anatomic landmarks identified. Nuclei are stained with DAPI (grey). Scale bars are 1 mm in (A) and 50 μm in (B).

hPGCs showed much lower levels of global DNA methylation, marked by anti 5-methylcytosine (5mC), than the surrounding somatic cells (Fig. 2A, Supplementary Fig. S2A), another striking feature distinguishing early hPGCs and somatic cells. The levels of 5-hydroxymethylcytosine (5hmC), generated by oxidation of 5mC (Ficz *et al.*, 2011; Hackett *et al.*, 2013) were also evaluated. Both early hPGCs and neighbouring somatic cells exhibited perinuclear foci of 5hmC (Fig. 2B, Supplementary Fig. S2B).

## POU5F1, NANOG and TFAP2C mark migratory and early colonizing hPGCs

Most migratory and early colonizing POU5F1+ hPGCs were also positive for other pluripotency markers, including NANOG, ALPL (or TNAP) and TFAP2C (or AP2γ) (Fig. 2C and D). ALPL was also present, albeit at lower level, in the neural tube (Fig. 2C). Interestingly, this is also observed in mouse embryos of comparable developmental stage (Kwong and Tam, 1984).

SSEA1 has been one of the markers (together with ITGB3) used to FACS-sort differentiated mPGCLCs from mPSCs (Hikabe *et al.*, 2016; Zhou *et al.*, 2016), therefore it was important to test its specificity in hPGCs. In agreement with Liu and colleagues (Liu *et al.*, 2004), we were unable to detect SSEA1 in paraffin sections of early hPGCs, but observed expression in parts of the mesonephros (Fig. 2D, Supplementary Fig. S3A). This contrasted with studies that showed SSEA1 in paraffin sections of human gonads from later developmental stages (Kerr *et al.*, 2008a, b; Park *et al.*, 2009).

We observed that the pluripotency marker SOX2 was absent from early hPGCs (Fig. 2D), as described for later stage hPGCs (Perrett *et al.*, 2008). However, prominent SOX2 staining marked the neural tube (Fig. 2D), confirming previous observations in human CS12 and CS16 (Olivera-Martinez *et al.*, 2012). Moreover, abundant SOX2 was observed in paraffin sections of teratomas derived from the embryonal carcinoma line 2102Ep (Supplementary Fig. S3B).

## Expression of mPGC-markers PRDM1, DPPA3 and IFITM3 in early hPGCs

Next, we investigated the expression of genes known to mark early mPGCs in mice (Saitou *et al.*, 2002; Ohinata *et al.*, 2005) and regularly used to access *in vitro* differentiation to hPGCLCs (Clark *et al.*, 2004; Bucay *et al.*, 2009; Kee *et al.*, 2009; Gkountela *et al.*, 2013; Irie *et al.*, 2015; Tilgner *et al.*, 2008; Sasaki *et al.*, 2015; Sugawa *et al.*, 2015). Specific nuclear PRDM1 was sufficient to identify POUF51+ hPGCs (Fig. 3A and B). The antibody used against DPPA3 showed higher expression in POUF51+ hPGCs, but the expression was restricted to the cytoplasm instead of being nuclear (Fig. 3A). IFITM3 showed low levels of expression overall in the embryo and did not mark hPGCs specifically (Fig. 3B). We further analysed the expression of DPPA3 and IFITM3 in OCT4+ hPGCs in older human embryos [week (W)8–9 of development] in both paraffin sections and whole mount and confirmed the cytoplasmic staining of DPPA3 in hPGCs and the aspecific staining of IFITM3 (Supplementary Fig. S4). Thus, we suggest caution when using DPPA3 and IFITM3 antibodies to identify hPGCLCs.

## Specific expression of SOX17, SALL4 and PDPN in early hPGCs

Recently, SOX17 has been shown to be expressed in hPGCs and to be a critical determinant during *in vitro* differentiation to both hPGCLCs fate (Irie *et al.*, 2015), endodermal fate (Wang *et al.*, 2011) and endothelial fate (Zhang *et al.*, 2017). We showed that SOX17 was expressed almost exclusively in POU5F1+ hPGCs and endothelial cells including the dorsal aorta, but unexpectedly not in the endoderm-derived GATA6+ gut (Fig. 3C). In addition, we investigated the expression pattern of SALL4, a novel determinant of mPGCs (Yamaguchi *et al.*, 2015) and showed that SALL4 was expressed almost exclusively in POU5F1+ hPGCs, (Fig. 3D). Furthermore, we report the specific expression of the surface marker PDPN in POU5F1+ hPGCs, but also in the neural tube (Fig. 3D).

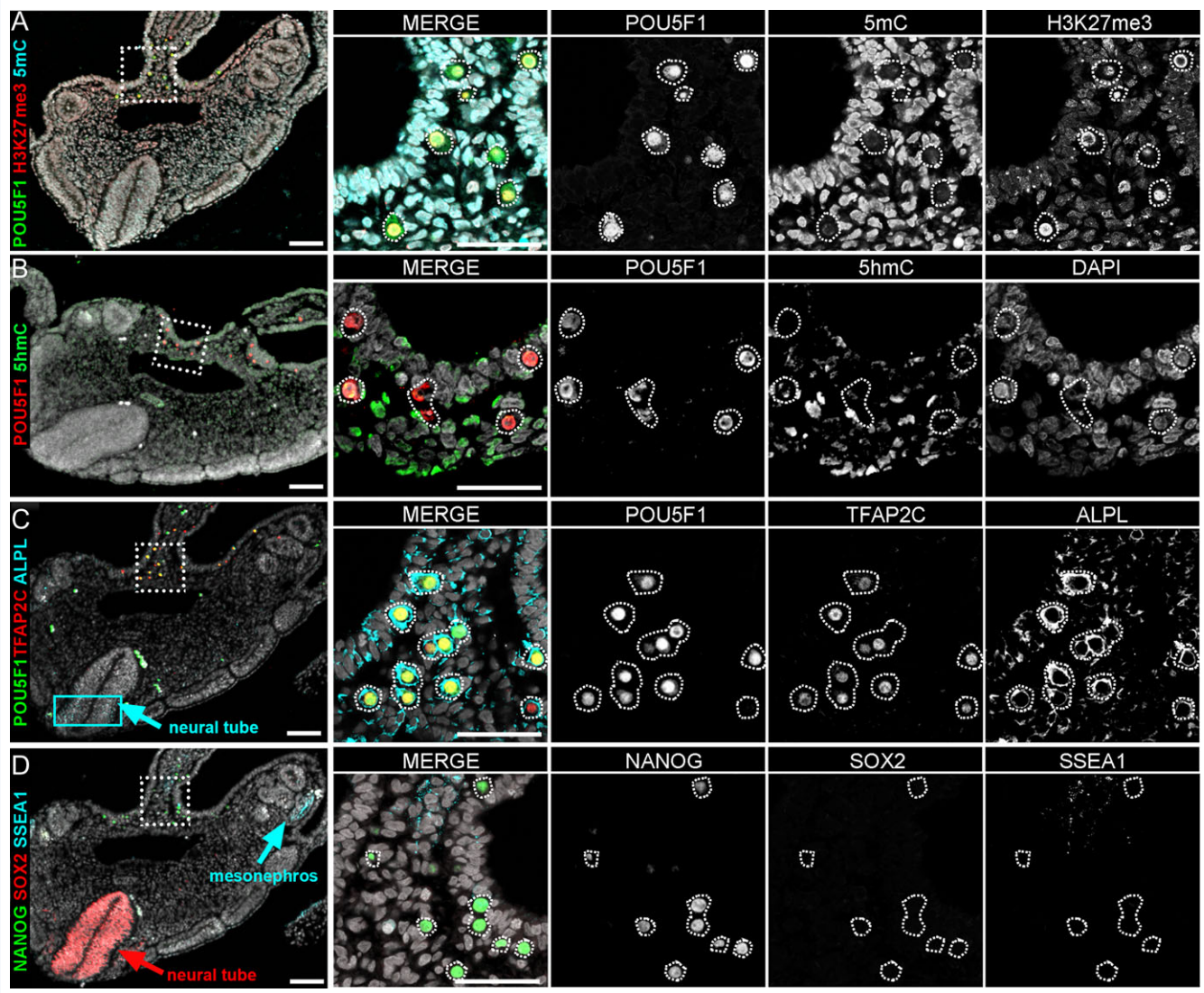


**Figure 2** Expression of pluripotency markers in the AGM of a CS12–13 human embryo. (**A**–**D**) Histological sections of the caudal part of the embryo immunostained for POU5F1 (green), H3K27me3 (red) and 5-methylcytosine (5mC, cyan) (A); POU5F1 (red) and 5-hydroximethylcytosine (5hmC, green) (B); POU5F1 (green), TFAP2C (red) and ALPL (cyan; cyan box depicts staining in neural tube) (C); and NANOG (green), SOX2 (red) and SSEA1 (cyan) (D). All sections were counterstained with DAPI (grey). Right panels depict a higher magnification corresponding to the dashed box in the left column (merge and single channels). Scale bars are 100 μm in the left column and 50 μm in all high magnifications.

## Expression of mesenchymal and adhesion molecules in early hPGCs

We detected a few T (or Brachyury)-positive cells among the POU5F1+ hPGCs (Fig. 4A). As expected, the notochord was strongly T-positive (Olivera-Martinez *et al.*, 2012). PECAM1 (or CD31) and CDH5 (or VE-Cadherin), surface markers of endothelial cells, marked both the dorsal aorta and blood capillaries, but were not expressed in POU5F1 + hPGCs (Fig. 4A and B). Blood capillaries, including those inside the glomeruli, in human kidneys at W16–18 were also positive for PECAM1 and CDH5 (Supplementary Fig. S3C and D).

Two other surface markers widely used to mark primed hPSCs, TRA-1–81 and SSEA4 (O'Connor *et al.*, 2008), were not expressed in POU5F1+ hPGCs on paraffin sections (Fig. 4B and C). At later stages, human gonads have been reported to show aspecific expression of SSEA4, but not TRA-1–81 (Kerr *et al.*, 2008a, b). As control for the TRA-1–81 and SSEA4 antibodies used, we showed that they marked cells in paraffin sections of teratomas derived from 2102Ep cells (Supplementary Fig. S3B) (Josephson *et al.*, 2007; Bouma *et al.*, 2017).

CDH1 (or E-Cadherin) regulates migration and homing of mPGCs (Richardson and Lehmann, 2010), whereas CDH2 (or N-Cadherin) is expressed in post-migratory mPGCs (Bendel-Stenzel *et al.*, 2000). Neither CDH1 nor CDH2 were expressed in POU5F1+ hPGCs (Fig. 4C and D), however, both were expressed in the surface ectoderm and additionally CDH1 marked the gut and mesonephros (Fig. 4C and D). As positive control, we showed that CDH1 marked the pseudostatified epithelium of collecting ducts in the renal pyramids

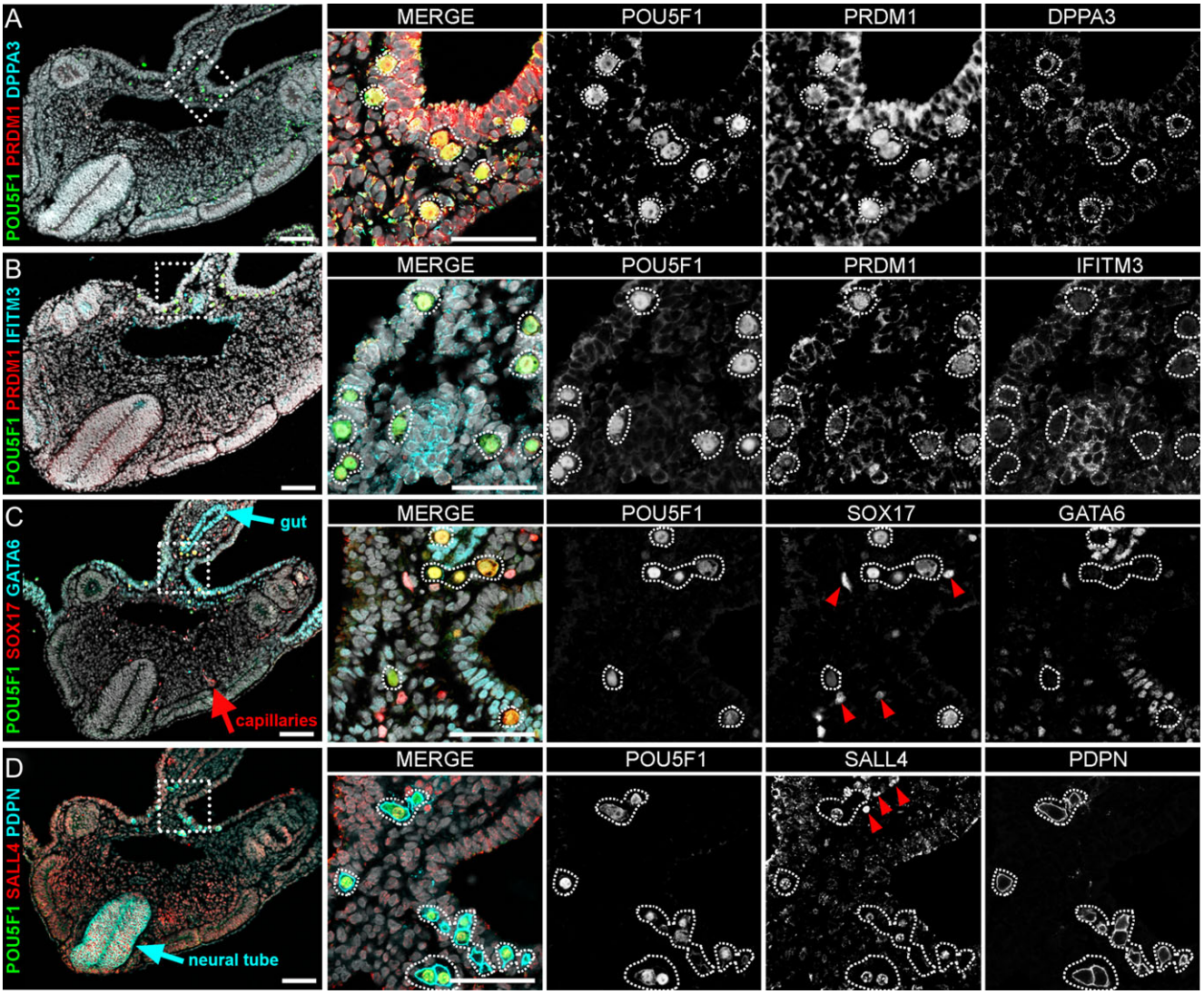


**Figure 3** Expression of germ cell-associated markers in the AGM of a CS12–13 human embryo. (**A**–**D**) Histological sections of the caudal part of the embryo immunostained for POU5F1 (green), PRDM1 (red) and DPPA3 (cyan) (A); POU5F1 (green), PRDM1 (red) and IFITM3 (cyan) (B); POU5F1 (green), SOX17 (red) and GATA6 (cyan) (C); and POU5F1 (green), SALL4 (red) and PDPN (cyan) (D). All sections were counterstained with DAPI (grey). Right panels depict a higher magnification corresponding to the dashed box in the left column (merge and single channels). Scale bars are 100 μm in the left column and 50 μm in all high magnifications.

and CDH2 marked convoluted tubules, most probably the proximal tubuli (Nouwen *et al.*, 1993) in the human kidney at W16 (Supplementary Fig. S3D).

TUBB3 was expressed in POU5F1+ hPGCs, as shown at later stages (Heeren *et al.*, 2016), but also marked the gut, the neural tube and interestingly the myotome (Fig. 4D). In conclusion, none of the mesenchymal or adhesion markers studied seemed specific enough to reliably identify POU5F1+ hPGCs, and hence hPGCLCs.

## Signalling pathways involved in the migration of hPGCs

Two chemoattractant cytokine–cytokine receptor systems known to be involved in human cancer, CXCL12/CXCR4 and KITLG/KIT (Teicher and Fricker, 2010; Salomonsson *et al.*, 2013), also regulate aspects of PGC migration in mice (Richardson and Lehmann, 2010). To understand whether these two molecular systems also regulate PGC migration in humans, we studied the expression of the cytokine receptors CXCR4 and KIT (or CD117). We did not observe expression of CXCR4 in POU5F1+ hPGCs on paraffin sections, but hPGCs showed expression of KIT (Fig. 5A and B). CXCR4 expression was confirmed in paraffin sections of W19 human adrenal and placenta (Supplementary Fig. S3E and F) (Fischer *et al.*, 2008).

Interestingly, KIT was highly expressed in a clump of cells located in the luminal–ventral side of the dorsal aorta (Fig. 5B), presumably bona-fide progenitors of hematopoietic stem cells that give rise to the adult hematopoietic system. Cells at this location were also positive for SOX17 (Fig. 3C), PECAM1 (Fig. 4A) and CDH5 (Fig. 4B); confirming

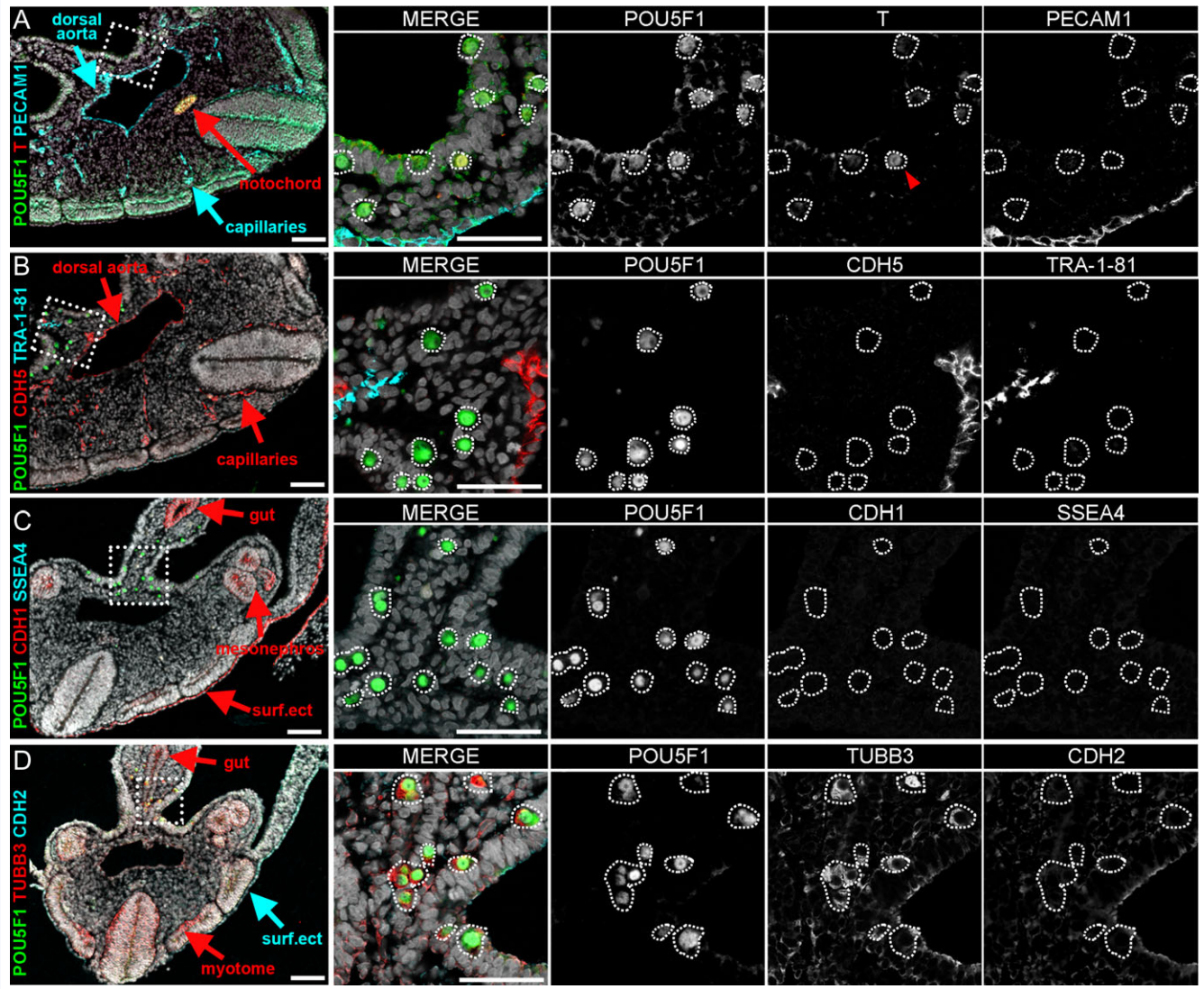


**Figure 4** Expression of mesenchymal and adhesion molecules in the AGM of a CS12–13 human embryo. (**A**–**D**) Histological sections of the caudal part of the embryo immunostained for POU5F1 (green), T (red) and PECAM1 (cyan) (A); POU5F1 (green), CDH5 (red) and TRA-1–81 (cyan) (B); POU5F1 (green), CDH1 (red) and SSEA4 (cyan) (C); and POU5F1 (green), TUBB3 (red) and CDH2 (cyan) (D). All sections were counterstained with DAPI (grey). Right panels depict a higher magnification corresponding to the dashed box in the left column (merge and single channels). Scale bars are 100 μm in the left column and 50 μm in all high magnifications. surf.ect, surface ectoderm.

their identity as foetal intra-aortic hematopoietic cell cluster (Ivanovs *et al.*, 2014; Nobuhisa *et al.*, 2014). In addition, KIT was also detected in the mesonephros, neural tube and dermatome (Fig. 5B).

In mice, ITGB1 (or integrin β1) is expressed in migratory PGCs (Anderson *et al.*, 1999), but in paraffin sections of the human AGM, the ITGB1 antibody showed faint ubiquitous staining (Fig. 5A). By contrast, ITGA6 (or integrin α6) showed specific expression in early hPGCs, the notochord, ventral part of the gut and surface ectoderm (Fig. 5C). ITGA6 and EPCAM were recently used to FACS-sort hPGCLCs differentiated from hPSCs (Sasaki *et al.*, 2015), however, in paraffin sections EPCAM only marked the gut where it colocalized with ITGA6 in the ventral part, but not the TFAP2C+ hPGCs (Fig. 5C). EPCAM expression was confirmed in paraffin sections of W19 human colon (Supplementary Fig. S3G) (Schnell *et al.*, 2013).

Finally, we tested CD38, surface marker used to isolate hPGCLCs from differentiating-hPSCs by FACS-sorting (Irie *et al.*, 2015), and observed cytoplasmic staining in hPGCs (Fig. 5D). PIWIL4 (Fig. 5D), a pre-meiotic PIWI-member (Siomi *et al.*, 2011) was enriched in small granules concentrated just outside the nuclear envelope in hPGCs, as observed at later stages (Gomes Fernandes *et al.*, 2018).

## Discussion

There is an increasing interest in the production of human gametes by *in vitro* differentiation of hPSCs. Currently, we either extrapolate knowledge from mouse early gametogenesis to understand the identity of hPGCLCs and/or compare hPGLCs to *in vivo* hPGCs using transcriptomics analysis (Clark *et al.*, 2004; Bucay *et al.*, 2009; Kee *et al.*,

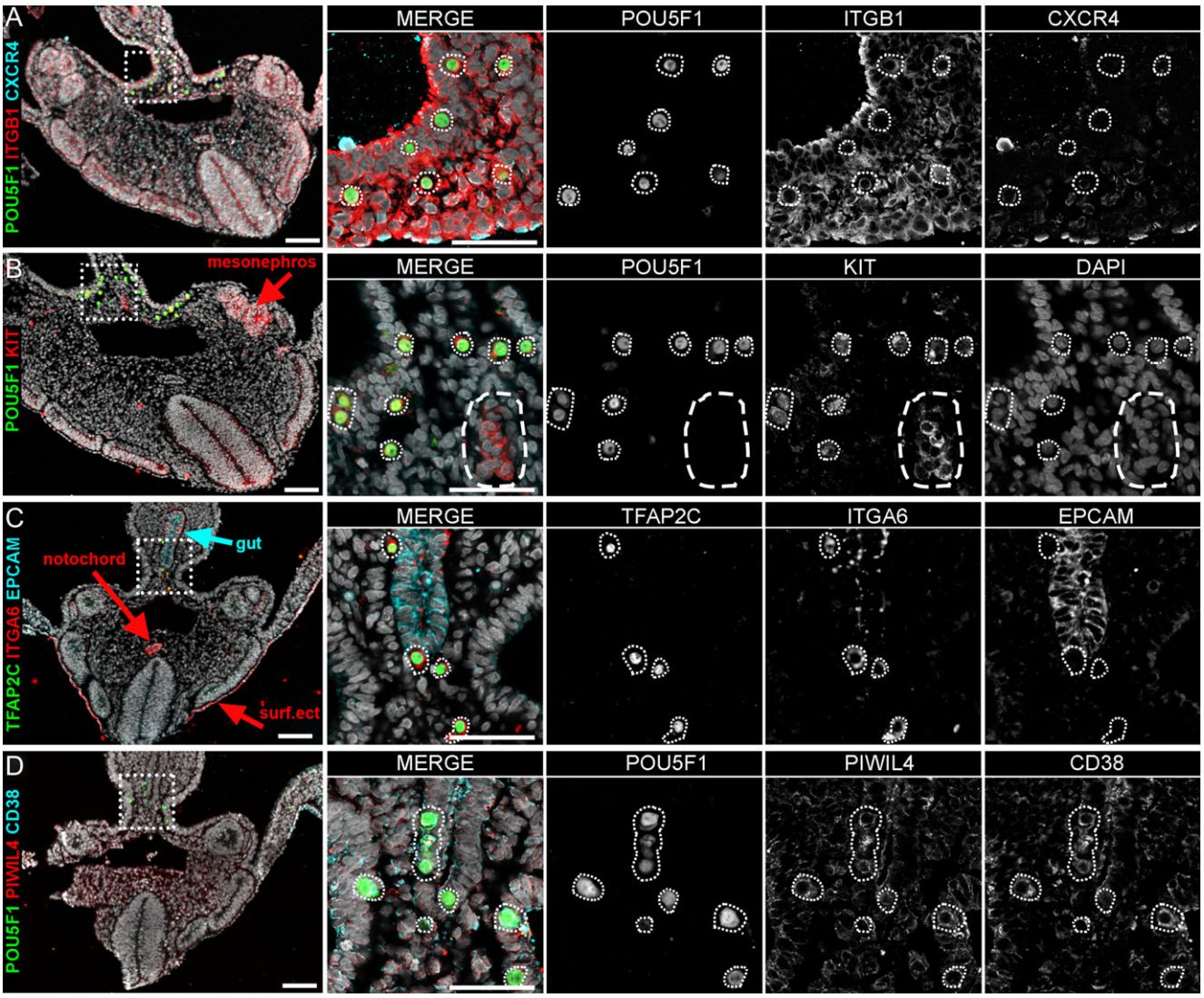


**Figure 5** Expression of migratory and surface markers in the AGM of a CS12–13 human embryo. (**A**–**D**) Histological sections of the caudal part of the embryo immunostained for POU5F1 (green), ITGB1 (red) and CRCXR4 (cyan) (A); POU5F1 (green), KIT (red) and DAPI (grey) (B); TFAP2C (green), ITGA6 (red) and EPCAM (cyan) (C); and POU5F1 (green), PIWIL4 (red) and CD38 (cyan) (D). All sections were counterstained with DAPI (grey). Right panels depict a higher magnification corresponding to the dashed box in the left column, as merge and each single channel except DAPI. Scale bars are 100 μm in the left column and 50 μm in all high magnifications. surf.ect, surface ectoderm.

2009; Gkountela *et al.*, 2013; Irie *et al.*, 2015; Tilgner *et al.*, 2008; Sasaki *et al.*, 2015; Sugawa *et al.*, 2015). Therefore, it is vital to have a robust toolbox of antibodies validated in migratory early hPGCs *in vivo* to evaluate and benchmark faithfully the different steps of gametogenesis, as well as to have reliable tools to isolate and purify hPGCLCs from other differentiated hPSCs in the dish.

We have tested a panel of 31 different markers (33 primary antibodies) in paraffin sections of the caudal part of a single human embryo (CS12–13), containing the AGM region, and determined their specificity to identify migratory and early colonizing POU5F1+ hPGCs. This study was limited by the fact that we analysed a single embryo, with a limited number of paraffin sections (and hence antibodies that we could test) using a single antigen retrieval method (citrate).

Using POU5F1 staining systematically allowed us to unambiguously identify early hPGCs, providing unique information regarding the specificity of the panel of 31 markers. Two different primary antibodies for POU5F1 were used and both showed high nuclear expression in hPGCs, but also cytoplasmic expression, characteristic of hPGCs at later stages (Gkountela *et al.*, 2013). Importantly, most antibodies corresponding to nuclear factors POU5F1, NANOG, TFAP2C and PRDM1 were sufficient to identify hPGCs, whereas SALL4 and SOX17 were specific to hPGCs but also recognized additional cell types in the AGM. The biological significance of the specific DPPA3 staining in the hPGCs cytoplasm remains to be investigated. Of note is the fact that PRDM14, a transcription factor necessary for mPGC specification (Yamaji *et al.*, 2008) and not tested in our study, was also shown to be

cytoplasmic in gonadal hPGCs (Irie *et al.*, 2015), suggesting that the DPPA3 staining pattern here observed may be of relevance.

The antibodies for the epigenetic marks H3K27me3 and 5mC (global DNA methylation) were sufficient to distinguish POU5F1+ hPGCs from the surrounding somatic cells. These two marks will be important to show whether differentiating hPGCLCs are undergoing correct reprogramming (von Meyenn *et al.*, 2016). Interestingly, the localization of H3K27me3 to the nuclear lamina of migratory hPGCs is similar to that in post-migratory gonadal (E11.5–E13.5) mPGCs (Prokopuk *et al.*, 2017) and different from that in migratory (E7.5–E9.5) mPGCs (Chuva de Sousa Lopes *et al.*, 2008). This species-specific difference is in agreement with the different dynamics regarding epigenetic remodelling observed in mice and humans (Gkountela *et al.*, 2015; Guo *et al.*, 2015; Tang *et al.*, 2016; von Meyenn *et al.*, 2016).

We were unable to detect IFITM3, SSEA1, SSEA4, TRA-1–81, CXCR4, CDH1, CDH2, CHD5, ITGB1 and EPCAM specifically in hPGCs in paraffin sections Nevertheless, SSEA1, CDH1, CDH2, CDH5, ITGB1 and EPCAM were not only expressed in other specific regions of the same paraffin section, but we also showed positive controls in paraffin-sections of human different tissues (teratoma, mesonephros, kidney, placenta, adrenal and colon). This suggested that mPGCs and hPGCs may respond to difference cues to migrate and hence express different surface markers, highlighting the need for functional studies and the validation of *in vitro* discoveries in the human.

The surface antibodies to detect ALPL, KIT and ITGA6 have been used successfully to isolate hPGCs (Gkountela *et al.*, 2013; Guo *et al.*, 2015) and/or hPGCLCs (Gkountela *et al.*, 2013; Irie *et al.*, 2015; Sasaki *et al.*, 2015; Sugawa *et al.*, 2015) by FACS. The surface marker PDPN may also be a suitable marker to include when identifying hPGCs and or hPGCLCs. However, we show here that those surface markers identified POU5F1+/TPAP2C+ hPGCs, but recognized other cell types in the caudal/AGM region. In the same line, cytoplasmic TUBB3 marked POU5F1+ hPGCs, but is also expressed in neural crest derivatives (Locher *et al.*, 2014; Heeren *et al.*, 2016) and other progenitor cell types such as the myotome. Therefore, we strongly suggest a combinatorial use of markers to unambiguously identify hPGCs or hPGCLCs.

We observed that several markers, such as KIT (Ivanovs *et al.*, 2014) and SOX17 (Zhang *et al.*, 2017), were expressed by both early POU5F1+ hPGCs and the intra-aortic hematopoietic stem cell cluster (luminal–ventral part of the dorsal aorta). Therefore, using these two markers alone may lead to the misidentification (or bulk isolation) of these two cell types. Interestingly, although IFITM3 showed no specificity for POU5F1+ hPGCs, we did notice an enrichment in the intra-aortic hematopoietic stem cell cluster (Fig. 3B). In mouse, IFITM3 has not been described as marker of intra-aortic hematopoietic stem cell clusters, but has been detected in (Runx1+) yolk sac hematopoietic cells (Mikedis and Downs, 2013).

We provide a unique insight in the specificity of a panel of 31 different markers, including pluripotency, surface and epigenetic markers, to identify and distinguish early hPGCs (and hPGCLCs) from the surrounding somatic cells. We report several striking differences between mPGCs and hPGCs and show that (surface) markers tend to react with several cell types in the embryo, including the intra-aortic hematopoietic stem cells present in the AGM. Transcription factors are usually not solely involved in the specification of a single lineage and cells can share the expression of many markers, hence, a careful and thoughtful choice of markers is crucial when studying *in vitro* differentiation. Our results provide a toolbox of markers to better evaluate protocols to induce the formation of hPGCLCs *in vitro*.

## Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online

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## Authors' roles

M.G.F., M.B., D.C.F.S. and S.M.C.d.S.L. designed the study, conducted experiments, analysed data and wrote the article. All authors approved the last version of the article.

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## Conflict of interest

The authors declare no conflict of interest.

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